# Effects of injection speed of test samples on the mouse bioassay for paralytic shellfish poisoning toxins

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## Abstract

The mouse bioassay has been used as the official method for paralytic shellfish poisoning toxins detection in Japan since 1980. However, differences in the results of this assay, when performed by different investigators, have been noted despite the use of the same sample. This study was performed to examine the effect of the injection speed, a hypothetical cause of such differences, on the death time of mice. Speed-controlled injection of the toxin (at 12, 6, 3, and 1.5 mL/min) into mice was performed using a syringe pump, and the death times of mice were measured. No statistically significant differences were found among the groups, even between fast injection (5 s) and very slow injection (40 s), indicating that the injection speed may not be the crucial factor for this assay.

## Introduction

The mouse bioassay (MBA) has been used as the official method for detecting paralytic shellfish poisoning (PSP) toxins in Japan since 1980 (Veterinary Sanitation Division, 1980). The MBA for PSP toxins is briefly as follows: five or more male ddY mice weighing 19-21 g (about 4 weeks of age) are intraperitoneally (ip) injected with 1 mL of acid extract of the shellfish sample, and the time of death (the time from the end of the injection to the last gasp of breath) is observed. If the median death time is <5 min, the assay is repeated by diluting the extract so that the animals die 5-7 min after injection. The toxicity of the sample, expressed in mouse unit (MU), is calculated from the median death time of total of 5 or more mice, by using Sommer's table (table for the relationship between death time and MU for PSP toxins). If mice weighing <19 g or >21 g are included in the assay, weight correction using a correction table for the weight of the mice is needed. The Japanese official method is basically conformed to the AOAC official method 959.08 (AOAC, 2005). In the AOAC official method 959.08, however, the use of a saxitoxin (STX) standard provided by the US Food and Drug Administration (FDA) as control is required every day or at least once a week, and toxicity is expressed by the STX equivalent (μg), instead of MU, which is calculated by using the result of STX standard because of the differences of mouse strain, gender, and conditions, for example. Moreover, the AOAC official method does not provide any detail regarding the requirements for mouse strain and gender. In Japan, however, the possession of STX is restricted by the Act on the Prohibition of Chemical Weapons and the Regulation of Specific Chemicals; therefore, the STX standard cannot be used in most facilities. Accordingly, in the Japanese official method, the strain and gender of the mice used are designated as an alternative to using the STX standard (Oshima, 2005).

Differences in the results of the MBA when performed by different investigators usually occurred, although the same sample was tested. Furthermore, the toxicity values obtained by inexperienced investigators, who have not been trained to handle mice, are often lower than those obtained by experienced investigators (personal communication). These differences among the results obtained by different investigators, hereafter referred to as *personal* differences, have shown similar trends almost every time; for example, the toxicity results determined by one researcher were almost always higher than those by another. These *personal* differences have also been seen in the results obtained using the STX standard according to the AOAC official method. In the AOAC official method, however, these *personal* differences are not problematic, because the results of the test samples are always compared with that of the STX standard. In contrast, in the Japanese official method, in which a standard is not used, these *personal* differences may be potentially problematic.In this study, we first showed examples of *personal* differences in the results of the MBA by different investigators. We then examined the effect of injection speed, a hypothetical cause of *personal* differences, on the time of death of the mice in the MBA.

## Materials and Methods

### Toxins

Saxitoxin (1 μg/mL) was provided by the US Food and Drug Administration (FDA) and used after 3-fold dilution, which was determined by a preliminary study.

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### Animals

Specific pathogen-free male ddY mice 4 weeks age were purchased from Japan SLC Inc. (Shizuoka, Japan) and kept in our animal facility for 1 day. The mice were kept at room temperature of 20-26°C and relative humidity of 30-70%, with a 12-h light-12-hour dark (9 a.m.-9 p.m. and 9 p.m.-9 a.m., respectively) cycle. The mice were housed in plastic cages with wood chip bedding and fed commercial pellets (CRF-1; Charles River Japan Inc., Kanagawa, Japan) and tap water *ad libitum*. All animal experiments were conducted with the approval of the Animal Care and Use Committee of the National Institute of Health Sciences, Japan (approval No.11, March 30, 2012).

### Mouse bioassay by two different investigators

The MBA method basically followed the Japanese official method (Oshima, 2005). Briefly, 3-fold diluted STX standard (1 mL) was ip injected into 5 male ddY mice weighing 19-21 g, and the times of death of the mice were recorded. The toxicity results (MU) were calculated using Sommer's table and the correction table for weight of the mice. This was carried out independently by 2 investigators. The examinations were performed in the same animal room at the same time. Diluted STX standard was prepared in 1 tube, mixed well, and used together by the 2 investigators. Mice were randomly used from the same cages. The examinations were performed twice. The 2 investigators had at least 15 years of experience of laboratory animal experiments and were well trained in the handling of mice.

### Effects of injection speed on mouse bioassay

The MBA method followed the Japanese official method (Oshima, 2005); however, some of the mice weighed 18.5-19 g, which was slightly lighter than the recommended weight. Speed controlled ip injections were performed using a syringe pump (FUSION100; CHEMIX Inc., Stafford, TX, USA), instead of manual injection. The injection times for 1 mL of inocula were set as 5, 10, 20, and 40 s, *i.e.* the injection speeds were 12, 6, 3, and 1.5 mL/min, respectively. The time of death was measured, and toxicity results (MU) were calculated by using Sommer's table and the correction table for the weight of the mice. The experiments were performed 3 times.

### Statistical analysis

The average and standard deviation (SD) of the median toxicity value of each injection speed of 3 experiments were statistically compared using the Student *t* test. Probabilities of less than 0.05 were considered significant.

## Results and Discussion

The results of the MBAs conducted by 2 different investigators are shown in Table 1. In both experiments, the toxicity values obtained by investigator 2 were higher than those obtained by investigator 1. The differences between the results obtained by the 2 investigators were approximately 5-10% of the MU, when compared with the median value, according to the official methods. Furthermore, the average values obtained by investigator 2 were higher than those obtained by investigator 1. However, no significant differences were found between the results obtained by the 2 investigators in both experiments. In this paper, only the results of 2 experiments are shown as examples; still, these trends are routinely found.

The time of death of the mice injected with different injection speeds are shown in Table 2 and the results of the statistical analyses of the 3 experiments are shown in Table 3. There were no statistically significant differences among the groups injected with the different injection speeds, even between fast injection (5 s) and very slow injection (40 s). The actual differences observed in the injection speeds of the 2 investigators were smaller than the differences in injection speeds in this study. Therefore, this indicates that the injection speed may not be the crucial factor for determining the time of death of the mice. It is thought that such *personal* differences may be produced by more complex and unquantifiable factors, but the reasons are not clarified in this study.

## Conclusions

To conclude, there is no standard used in the Japanese official method, as mentioned above. At present, decarbamoyl STX (dcSTX) – one of the derivatives of STX – is being considered for use as a standard instead of STX (Oshima, 2009). The usage of dcSTX as a standard would help to minimise individual differences in mice, together with the *personal* differences discussed in this paper.



**Table 1. Mouse bioassay for saxitoxin performed by two different investigators.**

| Mouse no. | BW (g) | BWCF | Lethal time (s) | MU | Corrected MU | Median MU | Average±SD of MU |
|---|---|---|---|---|---|---|---|
| **Experiment 1** | | | | | | | |
| Operator 1 | | | | | | | |
| 1 | 19.61 | 0.988 | 314 | 1.836 | 1.815 | 1.611 | 1.581±0.168 |
| 2 | 19.83 | 0.995 | 353 | 1.628 | 1.620 | | |
| 3 | 19.19 | 0.976 | 419 | 1.393 | 1.359 | | |
| 4 | 20.34 | 1.010 | 389 | 1.484 | 1.499 | | |
| 5 | 20.23 | 1.007 | 360 | 1.600 | 1.611 | | |
| Operator 2 | | | | | | | |
| 1 | 20.73 | 1.022 | 418 | 1.395 | 1.426 | 1.682 | 1.640±0.172 |
| 2 | 19.25 | 0.978 | 302 | 1.908 | 1.865 | | |
| 3 | 19.45 | 0.984 | 330 | 1.740 | 1.711 | | |
| 4 | 19.56 | 0.987 | 376 | 1.536 | 1.516 | | |
| 5 | 20.65 | 1.020 | 348 | 1.650 | 1.682 | | |
| **Experiment 2** | | | | | | | |
| Operator 1 | | | | | | | |
| 1 | 19.32 | 0.980 | 405 | 1.430 | 1.401 | 1.502 | 1.569±0.173 |
| 2 | 19.87 | 0.996 | 311 | 1.854 | 1.847 | | |
| 3 | 19.27 | 0.978 | 348 | 1.650 | 1.614 | | |
| 4 | 19.00 | 0.970 | 373 | 1.548 | 1.502 | | |
| 5 | 19.26 | 0.978 | 381 | 1.516 | 1.482 | | |
| Operator 2 | | | | | | | |
| 1 | 19.63 | 0.989 | 356 | 1.616 | 1.598 | 1.660 | 1.653±0.113 |
| 2 | 19.67 | 0.990 | 383 | 1.508 | 1.493 | | |
| 3 | 19.53 | 0.986 | 327 | 1.758 | 1.733 | | |
| 4 | 19.31 | 0.979 | 339 | 1.695 | 1.660 | | |
| 5 | 19.64 | 0.989 | 320 | 1.800 | 1.781 | | |

BW, Body weight; BWCF, body weight correction factor; MU, mouse unit; SD, standard deviation.

**Table 2. Lethal time after saxitoxin injection and different injection speeds.**

| Injection time (s) | Mouse no. | BW | BWCF | Lethal time (s) | MU | Corrected MU | Median MU | Average±SD of MU |
|---|---|---|---|---|---|---|---|---|
| **Experiment 1** | | | | | | | | |
| 5 | | | | | | | | |
| | 1 | 19.64 | 0.989 | 439 | 1.339 | 1.325 | 1.383 | 1.444±0.158 |
| | 2 | 20.09 | 1.003 | 408 | 1.422 | 1.426 | | |
| | 3 | 19.29 | 0.979 | 418 | 1.395 | 1.366 | | |
| | 4 | 19.70 | 0.993 | 332 | 1.730 | 1.719 | | |
| | 5 | 20.08 | 1.002 | 424 | 1.379 | 1.383 | | |
| 10 | | | | | | | | |
| | 1 | 19.44 | 0.983 | 327 | 1.758 | 1.728 | 1.728 | 1.736±0.203 |
| | 2 | 19.19 | 0.976 | 294 | 1.968 | 1.920 | | |
| | 3 | 19.81 | 0.994 | 349 | 1.645 | 1.636 | | |
| | 4 | 19.66 | 0.990 | 393 | 1.470 | 1.455 | | |
| | 5 | 20.45 | 1.014 | 301 | 1.914 | 1.940 | | |
| 20 | | | | | | | | |
| | 1 | 19.27 | 0.978 | 413 | 1.409 | 1.378 | 1.728 | 1.650±0.214 |
| | 2 | 19.86 | 0.996 | 389 | 1.484 | 1.478 | | |
| | 3 | 20.26 | 1.008 | 307 | 1.878 | 1.893 | | |
| | 4 | 20.38 | 1.011 | 328 | 1.752 | 1.772 | | |
| | 5 | 19.54 | 0.986 | 328 | 1.752 | 1.728 | | |
| 40 | | | | | | | | |
| | 1 | 20.20 | 1.006 | 344 | 1.670 | 1.680 | 1.680 | 1.790±0.259 |
| | 2 | 19.57 | 0.987 | 357 | 1.612 | 1.591 | | |
| | 3 | 20.04 | 1.001 | 270 | 2.160 | 2.163 | | |
| | 4 | 19.36 | 0.981 | 291 | 1.992 | 1.954 | | |
| | 5 | 19.32 | 0.980 | 361 | 1.596 | 1.563 | | |
| **Experiment 2** | | | | | | | | |
| 5 | | | | | | | | |
| | 1 | 19.80 | 0.994 | 387 | 1.492 | 1.483 | 1.624 | 1.643±0.138 |
| | 2 | 19.48 | 0.984 | 348 | 1.650 | 1.624 | | |
| | 3 | 19.03 | 0.971 | 311 | 1.854 | 1.800 | | |
| | 4 | 18.77 | 0.961 | 359 | 1.604 | 1.541 | | |
| | 5 | 18.83 | 0.963 | 314 | 1.836 | 1.768 | | |
| 10 | | | | | | | | |
| | 1 | 19.56 | 0.987 | 429 | 1.366 | 1.348 | 1.598 | 1.609±0.181 |
| | 2 | 19.12 | 0.974 | 310 | 1.860 | 1.811 | | |
| | 3 | 18.93 | 0.967 | 361 | 1.596 | 1.544 | | |
| | 4 | 19.41 | 0.982 | 324 | 1.776 | 1.745 | | |
| | 5 | 18.74 | 0.960 | 345 | 1.665 | 1.598 | | |
| 20 | | | | | | | | |
| | 1 | 18.80 | 0.962 | 378 | 1.528 | 1.470 | 1.533 | 1.594±0.205 |
| | 2 | 19.37 | 0.981 | 303 | 1.902 | 1.866 | | |
| | 3 | 19.72 | 0.992 | 328 | 1.752 | 1.737 | | |
| | 4 | 19.01 | 0.970 | 365 | 1.580 | 1.533 | | |
| | 5 | 18.61 | 0.954 | 406 | 1.427 | 1.362 | | |
| 40 | | | | | | | | |
| | 1 | 19.48 | 0.984 | 347 | 1.655 | 1.629 | 1.629 | 1.643±0.208 |
| | 2 | 19.59 | 0.988 | 325 | 1.770 | 1.748 | | |
| | 3 | 18.73 | 0.959 | 368 | 1.568 | 1.504 | | |
| | 4 | 18.97 | 0.969 | 400 | 1.447 | 1.402 | | |
| | 5 | 18.82 | 0.963 | 289 | 2.008 | 1.933 | | |
| **Experiment 3** | | | | | | | | |
| 5 | | | | | | | | |
| | 1 | 19.05 | 0.972 | 334 | 1.720 | 1.671 | 1.534 | 1.552±0.099 |
| | 2 | 19.81 | 0.994 | 354 | 1.624 | 1.615 | | |
| | 3 | 20.13 | 1.004 | 415 | 1.403 | 1.408 | | |
| | 4 | 19.34 | 0.984 | 370 | 1.560 | 1.534 | | |
| | 5 | 20.03 | 1.011 | 381 | 1.516 | 1.533 | | |
| 10 | | | | | | | | |
| | 1 | 19.46 | 0.984 | 363 | 1.588 | 1.562 | 1.653 | 1.643±0.076 |
| | 2 | 19.36 | 0.981 | 322 | 1.788 | 1.754 | | |
| | 3 | 20.09 | 1.014 | 370 | 1.560 | 1.581 | | |
| | 4 | 19.96 | 0.999 | 347 | 1.655 | 1.653 | | |
| | 5 | 19.76 | 1.000 | 345 | 1.665 | 1.666 | | |
| 20 | | | | | | | | |
| | 1 | 19.31 | 0.982 | 378 | 1.528 | 1.501 | 1.701 | 1.754±0.220 |
| | 2 | 19.70 | 0.991 | 351 | 1.636 | 1.621 | | |
| | 3 | 20.31 | 1.009 | 307 | 1.878 | 1.895 | | |
| | 4 | 19.72 | 0.992 | 335 | 1.715 | 1.701 | | |
| | 5 | 19.99 | 1.010 | 286 | 2.032 | 2.052 | | |
| 40 | | | | | | | | |
| | 1 | 19.87 | 0.996 | 403 | 1.437 | 1.431 | 1.643 | 1.699±0.220 |
| | 2 | 19.36 | 0.981 | 359 | 1.604 | 1.573 | | |
| | 3 | 20.17 | 1.017 | 356 | 1.616 | 1.643 | | |
| | 4 | 19.81 | 1.002 | 304 | 1.896 | 1.901 | | |
| | 5 | 19.40 | 0.986 | 293 | 1.976 | 1.948 | | |

BW, Body weight; BWCF, body weight correction factor; MU, mouse unit; SD, standard deviation.

**Table 3. Median mouse unit and median conversion factor in the three experiments.**

| Injection time (s) | Experiment 1 | Experiment 2 | Experiment 3 | Average±SD |
|---|---|---|---|---|
| 5 | 1.383 | 1.624 | 1.534 | 1.514±0.122 |
| 10 | 1.728 | 1.598 | 1.653 | 1.660±0.066 |
| 20 | 1.728 | 1.533 | 1.701 | 1.654±0.105 |
| 40 | 1.680 | 1.629 | 1.643 | 1.651±0.026 |

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